ASSESSMENT OF CERTAIN ENVIRONMENTAL AGENTS FOR MUTAGENICITY AND THE POSSIBLE ROLE OF GENOPROTECTIVE EFFECT OF NUTRACEUTICALS

Pre-Thesis Submission Colloquium Report

Submitted
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Abbreviations used in the report

VC : Vitamin C

CAT : Catalase

CFU : Colony forming units

CP : Cyclophosphamide

CPCSEA: The Committee for the Purpose of Control and Supervision of Experiments on Animals

DMSO : Dimethyl sulfoxide

EERM : Ethanolic extract of *Roccella montagnei*

GSH : Reduced glutathione

HPMC : Hydroxypropyl methylcellulose

L-AA : L-Ascorbic acid

LPO : Lipid peroxidation

MN(NCE): Micronucleated NCE

MN(PCE): Micronucleated PCE

MN : Micronucleus

MP : Methyl parathion

NCE : Normochromatic erythrocytes

NP : Nanoparticle

P/N : PCE/NCE

PCE : Polychromatic erythrocytes

ROS : Reactive oxygen species

SOD : Superoxide dismutase

ZnO NPs: Zinc Oxide nanoparticles

CHAPTER – I

Introduction and Review of Literature

1.1.Introduction

The industrial revolution has undoubtedly led to the rapid increase in the comfort of human lives; thus, providing an enormous number of benefits to society. The rapidly developing industrial sector has led to the use and release of many toxic chemicals into the environment. Many of the chemical agents released into the environment enter the body and cause adverse health effects. Humans are directly or indirectly exposed to these industrial chemicals via polluted air, food, and water. Not only human beings but also plants and animals get exposed to various pollutants either directly or via the food chain. Many pollutants that enter the biological system may induce mutation and associated health disorders (Gebhart, 1990; Weinstein *et al*., 1993; Nikiforov, 2004; Bukhari *et al*., 2006, Miranda *et al*., 2006; Godschalk *et al*., 2020). Several in-vitro and in-vivo tests conducted to assess the environmental agents' mutagenic potential have proved to be positive (Naik and Vijayalaxmi, 2009; Kayumov *et al*., 2019).

1.2. Scope of Toxicology

Toxicology is a discipline that essentially deals with the study of the potential of chemicals or a mixture of them, to produce harmful effects in living organisms and determining the implications of these effects (Bukhari *et al*., 2006). Experimental point of view, toxicology is a study of the interaction between chemicals and biological systems in order to quantitatively determine the potentiality of chemicals to produce injury resulting in adverse effects on living entities and to investigate the nature, incidence, mechanism of action, factors influencing their development, and reversibility of such adverse effects.

The toxicity of various agents to eukaryotic organisms can be classified into two broad categories, i.e., organ toxicity and genotoxicity. Genotoxicity is the implication of toxic effects at cellular and nuclear levels. The agents that produce alterations in nucleic acid and associated components at subtoxic exposure levels, leading to modified hereditary characteristics or DNA damage are classified as genotoxic agents (Brusick, 1987).

1.3. Review Literature

1.3.1. Importance of Genotoxicity Assessment

Genetic toxicology gained recognition as a formal discipline in the year 1969. It is regarded as an important field in toxicology with its prime aim being the identification of the toxic agents capable of causing the genetic and somatic mutations with their corresponding carcinogenic properties (Phillips and Arlt, 2009). The severe impact that these genotoxic agents impose on living organisms can be very subtle and irreversible, that includes birth defects and heritable genetic diseases including the development of cancers (Preston and Hoffmann, 2008; Kayumov *et al*., 2019).

The genotoxic effects of different environmental agents on the genetic materials of the organisms have evolved as a separate discipline of toxicology and it is known as Genetic Toxicology / Genotoxicology (Tsatsakis *et al*., 2017). Being an applied science, Genotoxicology depends on several basic sciences such as Genetics, Cytology, Chemistry, and Pharmacology, Statistics and many branches of Biology.

A major objective of biological sciences and environmental research is identifying and assessing the effects of environmental exposures on human health. The identification of exposure to chemical agents that produce mutations is an important task for risk assessment and prevention (Wogan, 1992). Recent shreds of evidence indicate that the molecular effect of the environment may extend beyond the interaction with the DNA sequence (Williams, 1989; Jackson and Bartek, 2009; Mansoori and Gautham, 2014; Balmain, 2020).

1.3.2. Genotoxicity with Special Reference to Nanoparticles:

Nanomaterials or nanoparticles can be defined as materials with an external dimension in the nanoscale or having an internal structure or surface structure in the nanoscale (Powers *et al*., 2006). The nanoscale is often considered to be 10 -100 nm. The special physicochemical properties of nanomaterials, improved functionalities, and leading performances make it a unique product of rapid manufacture (Karimi *et al*., 2016). At the same time, there is currently a concern regarding the potentially hazardous effects of nanomaterials on human health (Lehner *et al*., 2019). In recent years, so much research attention has been given to evaluating the mutagenicity/genotoxicity of nanoparticles. Based on earlier reports, it has been proposed that nanoparticles induce mutation through two pathways; the direct pathway which is based on the direct interaction of nanoparticles with genomic materials, while the indirect pathway is through the nanoparticles-mediated generation of ROS including hydroxyl, peroxide, and superoxide radicals (Barabadi *et al*., 2019). There are reports on the mutagenicity of various nanoparticles in a battery of in vitro and in vivo systems (Asharani *et al*., 2009; Wani and Shadab, 2020).

1.3.3. Role of Nutraceuticals Protecting against Genotoxic Agents:

Though many harmful chemical agents enter biological systems, it is not possible to completely avoid exposure to such agents. However, it should not be at the cost of health. There is a need for alternative solutions to avoid or minimize health risks caused by such agents. One approach given much priority in recent years is the usage of nutraceuticals. Nutraceuticals are products, in addition to their nutritional role are also used as medicine. By definition, a nutraceutical is a substance that has physiological benefits or provides protection against chronic disease (Nasri *et al*., 2014). In recent years, nutraceuticals have received significant attention owing to their potential nutritional, safety, and therapeutic effects (Rajasekaran *et al*., 2008; Nasri *et al*., 2014). In the present study, L-ascorbic acid and rutin were selected as nutraceuticals to evaluate their possible protective effect against mutagenicity/genotoxicity induced by the test chemicals.

1.3.3.1. Role of L-Ascorbic acid as a Nutraceutical:

Vitamins are essential nutrients required for various biochemical and physiological processes in the body. Most vitamins cannot be synthesized in the body and hence an external supplementation of vitamins via diet is essential. As far as human nutrition is concerned, vitamin C (L-ascorbic acid) is one such vitamin. It exists in reduced and oxidized forms as dehydroascorbic acid which is essentially inter-convertible and biologically active. Since its discovery about 75 years ago, vitamin C (VC) is known to be a ‘wonder worker in the biological systems (Aly and Donya, 2002). In addition to its role in collagen formation and other life-sustaining functions, it serves as a key immune system nutrient and a potent free-radical scavenger. It takes an active part in tissue metabolism and is connected with numerous electron-transport processes, where it behaves as a strong reducing agent. L-ascorbic acid as an antioxidant protects the body from various deleterious effects of free radicles, pollutants, and toxins (Odin, 1997; Vijayalaxmi and Venu, 1999; Antunes *et al*., 2000; Aly and Donya, 2002; Nefic, 2021).

Antimutagenicity of vitamin C against mutagenic complex mixture has been elucidated in *S. typhimurium* strain TA98 (Ong *et al*., 1989). Gentile *et al*. (1998) demonstrated antigenotoxic properties

of certain antioxidants including VC against the genotoxicity of anticancer drugs such as bleomycin, cisplatin, cyclophosphamide, and doxorubicin using *Salmonella*/microsome bioassay. Thus, there is ample experimental and epidemiological evidence in support of genoprotective effect of VC against a wide variety of agents.

1.3.3.2. Rutin and its Nutritional Significance:

Dietary manipulation can be regarded as a plausible approach to minimize genotoxicity. It is a worldwide accepted non-invasive strategy to mitigate the effect of genotoxic agents (Liu *et al*., 1992). One crucial antioxidant agent regarded as a potential portative measure against toxic agents is rutin. Rutin (3,3′,4′,5,7-pentahydroxyflavone-3- rhamnoglucoside) is a polyphenolic flavonoid present in many plants and is found in many food products like coffee, grains, fruits, and vegetables (Manach, 2005; Scholz, 2007; Ganeshpurkar and Saluja, 2017). Rutin is used in multiple commercial multi-vitamin preparations like influnet, antigrippin, and wobenzym (Mashkovskii, 2007). Harborne (1986) advocated that it is a vital nutritional component of foodstuff.

The modulating effect of rutin has been reported in earlier studies against various mutagenic agents (Francis *et al*., 1989; Snijman *et al*., 2007; Mladenovic *et al*., 2013; Khan *et al*., 2018; Ashoka and Mustak, 2019). In the present study, an attempt was made to study themutagenicity of the selected chemicals and the antimutagenic effect of nutraceuticals, viz., Rutin and L-Ascorbic acid.

1.3.4. *Roccella montagnei* – a natural source of nutraceuticals

Natural sources particularly plant, algal and fungal extracts have been given much research attention these days to evaluate their antimutagenic and anticarcinogenic effects. It is well-known that they are good sources of various nutrients, including antioxidants. Owing to being composed of many nutrients, it is expected that crude extracts of natural sources may significantly impart the synergistic effects to reduce toxic insults caused by chemical exposures (Namiki, 1990). In this context, an attempt was also made to evaluate the possible antimutagenic activity of ethanolic extract of a lichen *R. montagnei* against sodium azide, a potent mutagenic agent by employing bacterial reverse mutation assay in *S. typhimurium* TA100 strain (Ames test).

1.3.5. In vitro and In vivo Experimental Protocols for Mutagenicity and Antimutagenicity of the Test Chemicals:

There are many in vitro and in vivo experimental protocols for the assessment of genotoxicity and the possible carcinogenic risks (Williams, 1989; Eastmond *et al*., 2009; Phillips *et al*., 2020). The assays included in the present study are well-accepted by regulatoryand advisory bodies as the standard and essential genotoxicity measurements. A brief accountof the importance of the experimental methods employed in the present study is given herewith.

Ames test: It is a widely employed protocol utilizing special strains of bacteria to test whethera chemical under study can induce mutation. A positive test in terms of a significant increasein the number of revertant colonies in response to test chemicals indicates that the chemical is mutagenic and therefore may act as a carcinogen. The method serves as a fast and suitable assay to estimate the mutagenic and carcinogenic potential of a compound. The procedure was described in the early 1970s by Bruce Ames and his group (Tejs, 2008). The assay has also been extended for antimutagenicity studies of various plant extracts and antioxidants against mutagenic agents (Cariño-Cortés *et al*., 2007; Priftis *et al*., 2018).

***In vivo* Experimental Model:** As our ultimate concern is human welfare, the experimental animal selected for (geno)toxicity studies should be closely related to the human species

to enablemeaningful extrapolation. Therefore, it is clearly of continuing importance for risk assessment purposes to conduct studies on laboratory animals to define the cytogen etic response followingacute and chronic chemical exposure (Tucker and Preston, 1996). Rodents, prim arily mice are extensively used for mutagenicity studies.

Bone marrow Micronucleus (MN) Assay: It is a cytogenetic method in toxicologicalscreening for the evaluation of agents for their genotoxic effects. The assay is recognized as one of the most successful and reliable assays for genotoxic carcinogens (Hayashi, 2016). Micronuclei are cytoplasmic chromatin bodies having the appearance of small nuclei. They arise from acentric chromosomal fragments or from lagging chromosomes at the anaphase stage. Thus, the frequency of occurrence of micronuclei in treated cells provides a comparatively rapid and sensitive indication of both chromosomal breakage and chromosomal loss that lead to numerical chromosomal anomalies. The rodent micronucleus assay can be used as a sensitive screen to assess the genotoxicity or for more extensive/qualitative hazard determinations to aid in risk assessment. These young erythrocytes are termed polychromatic erythrocytes (PCE) and are distinguished from the mature normochromatic erythrocytes (NCE) by their differential staining properties. With a combination of Giemsa and May-Gruenwald staining, the PCEs stain bluish to purple due to their high content of RNA in the cytoplasm. In contrast,the NCEs stain reddish to yellow and are also slightly smaller than PCEs (Adler, 1984).

Sperm Abnormality Assay: It is essential to study the genotoxicity of the chemicals not only on somatic cells but also in germinal cells. Because, germ cell mutagens in addition to causing reproductive-related problems in the exposed individuals, will carry their effects to the offspring. Mammalian spermatogenesis is highly sensitive to ionizing radiations and certain chemicals (Johannisson *et al*., 2003). Therefore, the studies on the testicular effects of these agents will be valuable for determining the spectrum of reproductive damage expected from chemical agents. Scoring of abnormal sperms among the populations of normal sperms gives an idea about the mutagenic potency of the test chemical. A physical or chemical agent that induces abnormal sperms is an agent that clearly interferes with the normal differentiation of germ cells (Wyrobek and Bruce, 1978). For several years, analysis of sperm morphology alongwith its count, and motility, as an endpoint is extensively being used for the evaluation of genotoxicity of chemicals and also for the study of genoprotective effects (Martin *et al*., 1980; Vega *et al*., 1988; Mathew *et al*., 1992; Rai and Vijayalaxmi, 2001; Vijayalaxmi and D'Souza, 2004; Narayana *et al*., 2005; Talebi *et al*., 2013; Shen *et al*., 2019).

Sperm Chromatin Dispersion (SCD) test: Spermatozoa from infertile men have a higher frequency of chromosomal abnormalities, poor DNA packing quality, increased DNA strand breaks, and susceptibility to drug-induced DNA denaturation in situ than spermatozoa from fertile men (Sharma *et al*., 2004). DNA damage in the male germline is associated with fertilization failure, impaired pre-and post-implantation development, and poor pregnancy outcome (Fernandez *et al*., 2003). It is one of the commonly employed methods to assess toxicity and genoprotective effect.

Positive Controls: The use of a positive control group is an integral part of all toxicity studies,both in vitro and in vivo. It is required to maintain minimum assay acceptance criteria, and ensure that all steps in an experiment have been followed precisely (Rollin, 2006). In the present study, cyclophosphamide (CP: $C_7H_{15}C_{l2}N_2O_2P.H_2O$) was used as a positive mutagen. CP is a well-known mutagen and carcinogen. Sodium azide (NaN_3) was used as the positive agent for the Ames test.

Following biochemical parameters to evaluate the free radical-mediated toxicities of the test chemicals and antioxidant potency of the nutraceuticals were employed in the presentstudy. Significant changes in the catalytic activities in response to the administered chemicals/antioxidants indicate the toxicities/protective effect of the test agents. The liver andtestes of the experimental animals were used for the determination of the detoxifying enzyme activities and lipid peroxidation.

Catalase activity: It is a detoxifying enzyme catalyzes the decomposition of hydrogen peroxide into water and oxygen. It is found in nearly all living organisms exposed to oxygen, including bacteria, plants, and animals. It protects the cell/ tissues from oxidative damage by reactive oxygen species (Sinha *et al*., 1972).

Superoxide dismutase (SOD) assay: There are many xenobiotics generate superoxides and cause damaging effects on biomolecules. SOD is an enzyme that catalyzes the dismutation of the superoxide (O^{-2}) radical into ordinary molecular oxygen (O_2) and hydrogen peroxide (H_2O_2). Superoxide is produced as a by-product of oxygen metabolism and, if not regulated, causes many types of cell damage (Beyer and Fridovich, 1987). The assay to quantify the ability of SOD to split the superoxides into less reactive oxygen species developed by Kakkar *et al*. (1984) was employed.

Reduced Glutathione (GSH) Assay: It is a tripeptide with a gamma peptide linkage between the carboxyl group of the glutamate side-chain and cysteine. Glutathione is capable of preventing damage to important cellular components caused by reactive oxygen species such as free radicals, peroxides, lipid peroxides, and heavy metals. The carboxyl group of the cysteine residue is attached by a normal peptide linkage to a glycine. GSH provides reducing equivalents to antioxidant enzymes, hydroxyl radicals (Ault and Lawrence, 2003).

Lipid Peroxidation: It is oxidative damage that affects cellular membranes, lipoproteins, and other molecules composed of lipids in response to oxidative stress. Lipid peroxidation is a chain reaction and is created by free radicals influencing unsaturated fatty acids in cell membranes, leading to their damage (Halliwell and Chirico, 1993). Lipid peroxidation assay is considered a simple but sensitive approach to evaluate the ROS-mediated toxicities of chemicals.

Histological studies: The microscopic observation and alteration of the gross structure of tissue subjected to think sectioning indicates the damage caused by the test chemicals. In toxicological research, histological studies have great importance to further understanding the mechanism of adverse effects (Scotter *et al*., 2003). In the present study, testes of the treated animals were dissected and analyzed for damaging effects induced by the test chemicals through histological slide preparation.

With the background knowledge and literature survey, the present work was taken up to evaluate certain environmental agents with special reference to nanoparticles (TiO_2 and ZnO nanoparticles) for their mutagenicity in somatic and germinal cells. The possible modulatory effects of nutraceuticals (L-ascorbic acid and rutin) against the selected test chemicals such as TiO_2 and ZnO nanoparticles, methyl parathion and sodium azide were carried out by employing the aforesaid standard experimental protocols.

1.4. Objectives of the Study:

1.To evaluate the dose and time yield mutagenic effects of a Zinc Oxide (ZnO) nanoparticle and modulatory effects of nutraceuticals (L-Ascorbic Acid and Rutin) in somatic and germinal cells.
2.To evaluate the mutagenic potency of Titanium Dioxide (TiO_2) nanoparticle and modulatory effects of nutraceuticals (L-Ascorbic Acid and Rutin)) in somatic and germinal cells.
3.To assess the mutagenicity of methyl parathion and the protective effect of the nutraceuticals using the Ames test.
4.To study the biochemical basis of protective action of the selected nutraceuticals.
5. To evaluate the antimutagenic activity of *Roccella montagnei* against a carcinogenic agent, sodium azide using the Ames test.

1.5. Significance of the Study:

When there is a health risk due to exposure to genotoxic agents, it is better to stop or restrict their production or reckless applications. In many cases, complete avoiding of certain agents is impossible, particularly for pharmaceutical and consumer products. An alternative but effective approach is the usage of protective agents of natural origin. Agents which have nutritional as well as medicinal significance are considered to be very useful in minimizing the toxicities induced by agents. The parameters which are used for genotoxicity assessment can also be employed for evaluations of the modulatory effects against genotoxic agents.

There are limited studies on the genotoxicity of nanoparticles and the genoprotective effects of nutraceuticals against them. The present study was able to generate some data regarding the genotoxicity of certain environmental agents and the role of nutraceuticals against their genotoxic effects.

CHAPTER – II

Evaluation of Mutagenicity of Zinc Oxide Nanoparticle and Modulatory Effects of Nutraceutical (L-Ascorbic Acid and Rutin)

2.1. Introduction and Review of Literature

In recent years, the potential industrial applications of various nanoparticles have led to an exponential increase in their demand. The high demand and widespread utility of nanoparticles in many consumer products resulted in constant exposure to such agents (Kuhlbusch *et al*., 2011; Turan *et al*., 2019). Unintentional and accidental exposures to nanoparticles or nano-particle-based consumer products may occur via inhalation,dermal exposure, and gastrointestinal tract absorption, which in turn affect health (Borm *et al*.,2006; Kreyling *et al*., 2006). However, the research information on their adverse effects/toxicities is very scanty despite their widespread usage and exposure. Therefore, toxicity studies of nanoparticles are very essential to give emphasis on their possible health risks.

Among various nanoparticles (NP), zinc oxide (ZnO NP) is a commonly employed NP owing to its many beneficial physicochemical properties. It has wide-range applications in various industrial and commercial products (Espitia *et al*., 2012; Sabir *et al*., 2014). ZnO NPs are used in consumer products such as cosmetics, paints, and also in some clinical purposes. Its therapeutic applications include an ointment for external antibacterial infection, lotions, mouthwashes, and surface coatings to prevent microbial growth (Jiang *et al*., 2018). ZnO-NPs have also been utilized in the food industry as dietary supplements in human and livestock feed taking into consideration that zinc can stimulate the immune system and act as an anti-inflammatory agent (Prasad 2008; Espitia *et al*., 2012). It is a good photocatalyst and it has gained much consideration owing to its property of degradation and complete mineralization in the environment. It is considered environmentally safe compared to other nanoparticles/photocatalysts (Yeber *et al*., 2000; Shen *et al*., 2008).

There are certain studies in vitro and in vivo indicating that ZnO-NPs are toxic to mammalian cells and are even more toxic than other nanoscale structures of metallic oxide (Vandebriel and De Jong, 2012; Sirelkhatim *et al*., 2015).

With special reference to the mutagenicity and carcinogenicity of ZnO NP, there were some studies carried out in different experimental models. ZnO NPs have been reported to impart cytotoxic

effects and programmed cell death in human skin melanoma cells (Alarifi *et al*., 2013). ZnO NP was found to induce the respiratory disease through the generation of ROS, DNA damage, and activation of the p53 signaling pathway indicating its role in carcinogenesis(Dhawan and Anderson, 2015). The ZnO NPs-induced mutagenicity has been reported in bacterial cells (Yoshida *et al*., 2009). Jain *et al*. (2019) demonstrated the gene mutation at the HGPRT locus and cell cycle arrest associated with apoptosis in V-79 cells induced by ZnO NPs.

Although there are some reports, a literature survey indicates that there is a scarcity of research information on the mutagenicity of ZnO NPs on somatic and germinal cells of mammalian systems. The studies on the possible protective effect of nutraceuticals against ZnO NP-induced toxicities with special reference to mutagenicity are very limited. Considering wide-spread usage and exposure to ZnO NP, the present study was taken upto evaluate the mutagenicity in an animal model and bacterial system, and also to make an attempt to assess the possible protective role of the selected nutraceuticals (L-ascorbic acid and rutin).

2.2.Materials and Methods:

2.2.1.Chemicals and Reagents

Zinc oxide nanopowder, <50nm particle size (ZnO; CAS No:1314-13-2) purchased from Sigma Aldrich, Bengaluru, India was used as the test chemical.

Sodium azide (NaN_3; CAS No. 26628-22-8) purchased from HiMedia Laboratories, Pvt. Ltd., Mumbai, India was used as the positive mutagen. All other chemicals and reagents used in the experiment were procured from Merck, SRL, Himedia, India.

2.2.2.Ames test

2.2.2.1.Bacterial Strains

Salmonella entrica typhimurium (strains: TA98; MTCC ACC. No.1251 and TA100; MTCC ACC. No. 1252) were procured from Microbial Type Culture Collection and Gene Bank, CSIR-Institute of Microbial Technology (IMTECH) Council of Scientific and IndustrialResearch (CSIR), Chandigarh, India. The strains were retrieved by culturing in Vogel-Bonner (*VB*) *salt solution.*

2.2.2.2 Preparation of mouse liver microsomal enzymes (S9 fraction)

A healthy mouse was treated with phenobarbital (0.1%) for seven days. On the 6^{th} day,the animal was starved and sacrificed by cervical dislocation on the seventh day. The liver collected was washed with 0.1M KCl and homogenized. The homogenate obtained was centrifuged at 9000g for 10 minutes (Hubbard et al., 1985). The supernatant collected was stored at -80^{o}C and used whenever required.

2.2.2.3. Bacterial mutagenicity assay

The tests for assessing the mutagenicity of ZnO NP were conducted by following the pre-incubation assay (Maron and Ames, 1983).

2.2.3. In vivo study:

2.2.3.1. Experimental animal

Swiss albino mice of *Mus musculus* species (2n=40) were used as the experimental animals. The mice were bred and maintained in the departmental animal house in accordance with the guidelines prescribed by CPCSEA, India (http://cpcsea.nic.in/WriteReadData/userfiles/file/SOP_CPCSEA_inner_page.pdf). They were housed in polypropylene shoebox-type cages, bedded with rice husk and maintained in an air-conditioned room at 25°C (±2°C) and relative humidity of 65±5%. Animals were fed with standard mice pellets (Gold Mohur, India) and water ad libitum. The adult animals of 8-10 weeks old weighing 25-30 grams male were used for the experiments. The maintenance of animals and experiments were carried out after approval from the Institutional Animal Ethical Committee (Cert. No. MU/AZ/504(a)IAEC/2015-16, dated 23.0.2015). Guidelines given by subject experts of the Institutional Animal Ethics Committee (IAEC) during the ethical clearance certificate were strictly followed for the present work.

2.2.3.2. Dose and Treatment schedule

The toxicity of ZnO NP using bone marrow MN test and for biochemical and epididymal sperm parameters were assessed by selecting 3 single doses viz., 50, 100, and 200mg/kg b. wt. Experiments were also conducted for a multiple-dose treatment (50 mg/kg b.wt.for 7 consecutive days) of ZnO NP. The dose selection of ZnO NP was done based on earlier reports on its pharmacokinetics, tissue distribution, and excretion (Baek *et al*., 2012; Choi *et al*., 2014).

The toxicities of the selected nanoparticle were assessed by constituting the experimental animal grou ps as follows:

Group I: Solvent control treated with HPMC
Group II: Low dose group - ZnO NP (50 mg/kg b.wt)
Group III: Median dose group - ZnO NP (100 mg /kg b.wt).
Group IV: High dose group - ZnO NP (200 mg/kg b. wt.)
Group V: Distilled water control for cyclophosphamide (CP)
Group VI: Positive control group - CP (50 mg/kg b. wt.)

The aforesaid groups for three-time intervals 24, 48, and 72 hours were maintained.

Similar treatment groups were maintained for sperm parameters at 35 days after the treatment. In order to minimize the number of animals, bone marrow MN test, antioxidant enzyme assays, and assay for sperm parameters were performed using the same animals for 24 hours time intervals. A total of 5 healthy male mice were used for each control and experimental g roup.

The following experimental groups were maintained for evaluating the modulatory effect of the nanoparticles against ZnO NP.
Group I: Animals treated with distilled water (solvent control for nutraceuticals)
Group II: Animals treated with L-ascorbic acid (200x2 mg/kg)
Group III: Animals treated with Rutin (200x2 mg/kg)
Group IV: Animals treated with ZnO-NP 200 mg/kg b. wt. (the values derived from the toxicitytests).
Group V: Animals treated with L-ascorbic acid (50 x 2 mg/kg) + ZnO-NP (200 mg/kg)
Group VI: Animals treated with L-ascorbic acid (100 x 2 mg/kg) + ZnO-NP (200 mg/kg)
Group VII: Animals treated with L-ascorbic acid (200 x 2 mg/kg) + ZnO-NP (200mg/kg)
Group VIII: Animals treated with Rutin (50 x 2 mg/kg) + ZnO-NP (200 mg/kg)
Group IX: Animals treated Rutin (100 x 2 mg/kg) + ZnO-NP (200mg/kg)
Group X: Animals treated Rutin (200 x 2 mg/kg) + ZnO-NP (200 mg/kg)

Group XI: Animals treated with L-ascorbic acid + Rutin (100+100) x 2 (Control)
Group XI: Animals treated with [L-ascorbic acid + Rutin (100+100) x 2] + ZnO-NP (200mg/kg) + [VC + Rutin (100+100)mg/kg x2]

2.2.3.3. Bone marrow Micronucleus Test:

After treatment with the test chemicals/control agents, bone marrow preparations were made by employing the modified method of Hayashi *et al*. (1984).

2.2.3.4. Epididymal Sperm Parameters:

In this assay, one post-treatment sampling time (35 days) was used taking into account that the germ cells that are exposed at the late spermatogonial stage to the chemical take five weeks to reach the cauda epididymis after undergoing a series of changes to produce the matured sperms. In addition, for a short-term duration, experiments for a time interval of 24-hour exposure to the chemical were also performed. The cauda epididymis was minced thoroughly and the suspension obtained was filtered through a muslin cloth to remove tissue debris. The filtered suspension was stained for about 20 minutes, with 1% aqueous eosin Y by taking the sperm suspension and staining in a 10:1 ratio. A drop of the sperm suspension was taken on a clean slide, smeared and air-dried. The air-dried slides were observed under the 40X objective of a compound microscope with a green filter. A total of 2,000 sperms per animal were analyzed from each treatment and control group for the presence of sperm shape abnormalities. Scoring of the abnormal sperms was done according to the criteria of Wyrobek and Bruce (1975).

Assessment of sperm count and motility formed the additional parameters in this test protocol. Sperm count was done with the help of a hemocytometer. An aliquot (0.05ml) from the sperm suspension (1ml) was removed before staining and diluted 40 times with PBS (1:40) and mixed thoroughly. A sample of the diluted sperm suspension was introduced into the Neubauer counting chamber, and the total sperm count in 8 squares of 1 mm^2 was determined and multiplied by 5×10^4 to calculate the number of sperm per epididymis (Vega *et al*., 1988).

2.2.3.5. Sperm Chromatin Dispersion (SCD) test (Fernandez *et al*., 2003):

Spermatozoa from infertile men have a higher frequency of chromosomal abnormalities, poor DNA packing quality, increased DNA strand breaks and susceptibility to drug-induced DNA denaturation in situ than spermatozoa from fertile men (Chen *et al*., 2004).

2.2.3.6. Biochemical Parameters:

a. Catalase activity (Sinha *et al*., 1972):

Catalase activity was performed by employing the method of Sinha *et al*. (1972) with slight modifications.

b. Superoxide dismutase (SOD) assay:

The ability of SOD to split the superoxides into less reactive oxygen species was assayed by adopting the modified protocol of Kakkar *et al*. (1984).

c. Reduced Glutathione (GSH) Assay:

GSH level in testes was determined by the modified protocol of Ellman, (1959).

d. Lipid Peroxidation Assay:

Oxidative damage induced by the test agents in the testes homogenate was determined by using the modified protocol of Buege and Aust (1978).

2.2.3.7. Histological study:

The testes were dissected out from the controlled and treated groups. Then, fixed in Bouin's fixative for 48 h and preserved in 70% alcohol until further processing. The tissues were serially dehydrated using graded alcohol and embedded in paraffin. Thin transverse and longitudinal sectioning of the embedded tissue was done. Then, stained with haematoxylin and eosin for histological evaluation.

2.2.3.8.Statistical analysis:

The data were analyzed using GraphPad INSTAT 3 statistical package (GraphPad Software Inc, San Diego, USA). The data represented the mean ±SD of the raw data. The validity of the data was analyzed using a one-way ANOVA test, by Dunnett's post hoc method. Differences were considered to be statistically significant at three levels $p< 0.05$; $p<0.01$ and $p<0.001$.

2.3. Results and Discussion:

2.3.1. Ames test

The current observation is parallel to the earlier reports (Yoshida *et al.*, 2009; Pan *et al*., 2010), wherein ZnO NP with or without metabolic activation was shown to be non-mutagenic in the Ames test in different strains. Based on earlier reports (Wang *et al*., 2014; Ghosh *et al*., 2016) the slight increase in the revertant colonies in the presence of the S9 fraction as observed in the present study may be attributed to ZnO NP-mediated free radicals since it was demonstrated to induce the generationof oxidative stress upon metabolism.

2.3.2. Bone Marrow Micronucleus Assay

The % frequencies of the MN (PCE) and total micronucleated cells [MN (PCE) + MN (NCE)] were significantly higher compared to the solvent control group at 24 hrs of exposure to ZnO NP (100 mg/kg, $p>0.05$ and 200 mg/kg, $p>0.01$). The lower dose did not significantly increase the micronucleated cells. At 48 hours ofthe time interval of ZnO NP exposure, only the highest dose imparted the genotoxicity in termsof micronucleated cells compared to the control group ($p<0.05$). ZnO NP was not found to induce the formation of micronucleated cells at later time intervals at a significant level. The multiple treatments of the selected lower dose of the ZnO NP (50 mg/kg for 7 days) resulted inthe induction of micronucleated cells (PCE and NCE) at a significant level compared to the control group ($p<0.01$).

The PCE/NCE ratio declined in a dose-dependent manner 24 hours after the treatment of ZnO NP. However, statistically, it was found to be significant at 100 mg/kg b. wt($p<0.05$) and 200 mg/kg ($p<0.01$) compared to the control group. The magnitude of the cytotoxic effect in terms of reduction in P/N was found to be reduced at 48 hours; statistically 100 and 200 mg/kg b. w. of ZnO NP induced the effect ($p<0.05$). At 72 hours time interval, a significant reduction was observed only at a higher dose of the ZnO NP ($p<0.05$) compared to the control. The sub-chronic treatment of the test chemical significantly reduced the P/N ratiocompared to the control ($p<0.01$).

The positive control cyclophosphamide (CP) showed the mutagenic and cytotoxic potency in terms of an increase in the frequency of micronucleated cells and reduction in the P/N ratio respectively which statistically deviate at a significant level compared to the corresponding solven t control group ($p<0.001$).

The induction of micronucleated cells as noted in the present study are contradictory toan earlier report where ZnO NP was shown to be negative in the bone marrow micronucleus test conducted on Swiss albino mice (Srivastav *et al.*, 2017). The contradiction may be due to the route of exposure; in our study, the selected dose of the ZnO NP was administered intraperitoneally whereas, in their study, it was the oral route of administration. The route of exposure is an important factor that plays a significant role in toxicities, including mutagenicity(Sekihashi *et al*., 2001; Tubaro *et al*., 2003). The Intraperitoneal route is more sensitive compared to the oral administration as depicted in the genotoxicity studies of some chemicals(Sekihashi *et al*., 2001). Our observation is in parallel with Srivastav *et al*. (2017); ZnO NP was shown to impart cytotoxicity in terms of reduction in the P/N ratio.

It was noted in the present study that at later time intervals (48 and 72 hours), there was a decline in the frequency of micronucleated cells and the P/N ratio. It has been shown that ZnO NPs at a concentration lesser than 300 mg/kg were distributed in tissues and excreted within 24 hours (Baek *et al*., 2012). The sub-chronic exposure of ZnO NP (50mg/kg for 7 consecutive days) resulted in a significant increasein the frequency of micronucleated cells and a reduction in the P/N ratio. This observation could be correlated to an earlier report (Chung *et al*., 2013), where it has been demonstrated that ZnO NPs accumulate in the blood circulation after repeated exposure.

It has been experimentally proven that cytogenetic damage and mitotic depression(reduction in P/N ratio) induced by various nanoparticles, including ZnO NP are connected with the production of reactive oxygen species (Sharma *et al*., 2012; Srivastav *et al*., 2017). In addition to the free radical medicated genotoxicity, it has also been proposed that nanoparticleslike ZnO NP can cross the cell membranes and diffuse into the nucleus or even it can be transported through the nuclear pore complexes. Upon reaching the nucleus they could directly interact with DNA in turn leading to DNA/chromosomal damaging effect (Magdolenova *et al*.,2013). The genotoxicity and cytotoxicity with special reference to the reduction in P/N ratio are closely interconnected which have been proven in earlier studies (D'Souza *et al*., 2014). Thus, the current observation of ZnO NP inducedNP-inducedgenotoxicity and cytotoxicity may be due todirect and indirect (free radical-mediated) effects in the somatic cells.

The results obtained for the evaluation of the effect of the Nutraceuticals on ZnO NP-induced bone marrow micronucleated cells and P/N ratio at 24 hr time intervals are presented in tabular forms. L-ascorbic acid and rutin at 200x2 mg/kg b. wt. (the maximum dose selected in the present study) individually and in combination (each 100x2 mg/kg b.wt) did not induce the formation of micronucleated cells and reduction in P/N ratio compared to the distilled water control group. Thus, the selected doses of the nutraceuticals are safe with reference to the genotoxicity and cytotoxicity in the Swiss albino mouse model. A dose-dependent reduction inthe frequency of micronucleated cells MN(PCE) was observed in the case of combination treatment with L-ascorbic acid at significant levels compared to the ZnO NP alone treated group (50x2 mg/kg, $p<0.05$; 100x2 mg/kg, $p<0.01$; 200x2mg/kg $p<0.001$. Ascorbic acid-induced the recovery of the ZnO NP induced reduction in the P/N ratio; however, a statistically significant increase in the ratio was observed only for the higher doses (100mg/kg, $p<0.5$; 200mg/kg, $p<0.01$).

The coadministration of rutin with ZnO NP resulted in a significant reduction in the frequency of micronucleated cells (MN-PCE and total) in a dose-depending manner (100mgx2, $p<0.05$; 200x2 mg/kg, $p<0.01$). The lower dose of rutin (50x2 mg/kg) was not to be statistically significant. The

enhancement of the P/N ratio was found to be statistically significant only in the case of the higher dose of rutin ($p<0.05$).

The combination of L-ascorbic acid and rutin each 100mgx2/kg weight and its treatment induced the ameliorating effect in terms of a significant reduction in micronucleated cells and enhancement of P/N ratio ($p<0.01$).

2.3.3. Epididymal Sperm Parameters

ZnO NP at its 24 hours post-treatment did not affect the morphology of spermatozoa at lower doses (50 and 100 mg/kg) compared to the solvent control group. The higher dose (200mg/kg b.wt) was found to mildly affect sperm morphology as indicated by the higher frequency of individual and total abnormal spermatozoa at a significant level ($p<0.05$) compared to the control group. The results of the sperm-chromatin dispersion test indicated that ZnO NP did not induce DNA damage at a significant level in the bone marrow cell of the experimental animal. The positive control (CP at 50 mg/kg b.wt.) induced sperm abnormalities and DNA damage was evident with a statistical significance ($p<0.01$) compared to the corresponding control group.

The data derived from the sperm chromatin dispersion test indicated that the test chemical did not induce DNA damage in the spermatozoa significantly unlike the positive control CP which induced a higher frequency of sperm with DNA damage at a significant level ($p<0.01$).

The results of the effect of ZnO NP induced on sperm morphology at 35-days post-treatment samplings are presented in the form of graphs. The % frequencies of individual type and total abnormal spermatozoa did not significantly deviate from the control values indicating that ZnO NP at its lower dose (50mg/kg b.wt.) was not toxic to spermatogenesis. There was a dose-dependent significant increase in the frequency of abnormal spermatozoa compared to the control group (100 mg/kg b.wt, $p<0.05$; 200mg/kg b.wt. $p<0.01$). The sub-chronic exposure of ZnO NP also induced sperm abnormality at a significant level ($p<0.01$) compared to its corresponding control group. The positive control cyclophosphamide (50mg/kg) significantly affected the sperm morphology in comparison with the solvent control group ($p<0.001$).

Spermatozoa with DNA damage were also in a higher frequency for the lower dose ($p<0.5$). However, the effect was not found to be statistically dose-dependent. The multiple treatments with ZnO NP resulted in DNA damage in spermatozoa at a significant level ($p<0.01$). The positive control CP-induced effect on sperm morphology and integrity of DNA was indicated by a statistically significant increase in the values compared to the control group($p<0.001$).

The values of the sperm chromatin dispersion test indicate that the ZnO NP at its lower dose did not induce DNA damage in the spermatozoa significantly. The significant effect was observed at higher doses ($p<0.05$); however, it was not dose-dependent. The multiple dosing of ZnO NP imparted the DNA damage in spermatozoa at a significant level compared to the control group ($p<0.01$). The positive control CP induced the higher frequency of sperm with DNA damage at a highly significant level ($p<0.001$).

The effect of ZnO NP on sperm count and motility at 24 and 37 days post-treatment sampling were shown in the form of bar diagrams. ZnO NP did not induce a reduction in sperm count and sperm

with progressive motility at all the tested doses at 24 hours time intervals, significantly. The positive control, cyclophosphamide-induced a significant reduction in the sperm count ($p<0.05$) as well as progressive motility ($p<0.01$). The 35 days post-treatment sampling induced a reduction in the sperm count and progressive motility at a significant level only at the higher dose of ZnO NP compared to the control group ($p<0.05$). Multiple dosing of animalswith ZnO NP (50mg/kg for 7 consecutive days) negatively affected the sperm count and motility as indicated by a significant reduction in the values compared to its corresponding control group ($p<0.01$).

Cyclophosphamide induced a significant reduction in sperm count and spermatozoa with progressive motility at a highly significant level at 35 days after the treatment compared to its control group ($p<0.001$).

The % frequency of total abnormal spermatozoa is significantly decreased in the animalgroup co-administered with the VC (fig.2.3.3.e). The lower dose of L-ascorbic acid induced the ameliorating effect with a statistical significance of $p<0.05$ whereas both of its higher doses induced the effect statistically at the same significant level ($p<0.01$) when compared with the ZnO NP alone treated group. This indicates that ascorbic acid (100 mgx2/kg b. wt) is the optimum dose yielding the maximum ameliorating effect against ZnO NP-induced sperm abnormality. The rutin also reduced the frequency of abnormal spermatozoa induced by ZnO NP at a significant level.

The lower dose of rutin induced the effect significantly ($p<0.05$) and the higher dose induced the effect significantly ($p<0.01$) compared to the ZnO NP alone treated group.

Thus, Rutin at its 100x2 can be considered the optimal dose in imparting the protective effect against ZnO NP-induced sperm abnormality. The combinationof ascorbic acid (100mg/kg) and rutin (100 mg/kg) and its treatment resulted in a significant decline in the frequency of abnormal spermatozoa compared to the ZnO NP (200mg/kg b. wt)treated group ($p<0.001$). This higher magnitude of reduction rate in the sperm abnormality compared to the individual treatment of ascorbic acid and rutin indicates their synergistic effect yielding the maximum protection against ZnO NP-induced toxicity.

The sperm chromatin dispersion test data indicates that the L-ascorbic acid reduced the ZnO NP-induced DNA damage in spermatozoa in a dose-dependent manner (50 mgx2 /kg b. wt. $p<0.05$; 100 mgx2 /kg b. wt. $p<0.01$; 200 mgx2 /kg b. wt., $p<0.001$) compared to the ZnO treated group (fig.2.3.3f). Recovery effect (decrease in the frequency of sperm with DNA damage compared to the ZnO NP alone treated group) was also observed in the case of the animals co-administered with rutin (50 mgx2 /kg b. wt. $p<0.05$; 100 mgx2 /kg b. wt. $p<0.01$).

However, the observed effects were not dose-dependent; the higher dose induced the recovery effect with the same magnitude. It indicates that rutin at its 100 mg/kg b. wt. impart the maximum modulatoryeffect against t he ZnO NP-induced DNA damage in spermatozoa. Further increase beyond thisdose was not found to have an additive effect. at its lower dose did not induce DNA damagein the spermatozoa significantly. The value of the control group treated with ZnO-NP (200) + [VC + Rutin (100+100)x2] indicates the protective effect of these nutraceuticals synergisticallywith a statistical significance ($p<0.01$) when compared with that of the ZnO NP alone treatedgroup. There was no significant induction of DNA damage by the nutraceuticals individually (200mg/kg b. wt.) and in combination [(VC + Rutin (100+100) x2], indicating that the selecteddoses are safe without causing any damaging effect in spermatozoa.

With reference to the sperm count and progressive motility, the differential values canbe seen with reference to the effects of the selected nutraceuticals on ZnO NP (fig.2.3.3.g and fig.2.3.3.h). L-ascorbic acid induced the recovery of sperm count and progressive motility only at its median and high doseswith the same statistical significance ($p<0.05$) when compared with the ZnO NP treated group.

There was not a dose-dependent effect indicating that L-ascorbic acid at its 100mg/kg b.wt. yielded the maximum protective effect against the count and motility.

Rutin induced the recovery of sperm count at its lower dose ($p<0.05$), but there was nostatistically significant increase in the count at its higher doses even at 200x2 mg/kg b. wt. ($p<0.05$). The coadministration with rutin induced the enhancement in the frequency of sperm with the progressive motility only at its higher dose with a statistical significance ($p<0.05$) compared to the ZnO NP treated group. The animals treated with the combination of rutin and L-ascorbic acid [ZnO NP (200)+[VC + Rutin (100+100)x2] enhanced the sperm count significantly ($p<0.001$) and progressive motility ($p<0.05$). Thus, L-ascorbic acid and rutin imparted some positive effects on sperm count and motility but with differential optimal doses. The combination of both the nutraceuticals was found to be effective in the recovery of sperm count rather than motility compared to their individual effects against ZnO NP.

The results obtained for the effect of nutraceuticals against ZnO NP-induced changes in enzyme activities (GSH, Catalase & SOD) and Lipid Peroxidation (LPO) were presented. The positive agent CP (50mg/kg b.wt) affected the liver and testes biochemical parameters by significantly deviating the values compared to the control group ($p<0.001$). The selected nutraceuticals L-ascorbic acid and rutin at 200x2mg/kg b. wt. (the maximum dose selected for the present study) individually and in combination (each 100x2 mg/kg b.wt) did not change the GSH, CAT, SOD, and LPO levels at a significant level compared to the distilledwater control group. Thus, the selected doses of the nutraceuticals are safe with reference to the biochemical parameters in the Swiss albino mice. The solvent control (HPMC) also did not significantly alter the biochemical parameters. The ZnO NP (200mg/kg b. wt)-treated group showed a significant decline in the GSH, CAT, and SOD activities and an increase in the LPOin both the tissue, liver and testes ($p<0.01$). The coadministration of ZnO NP with L-ascorbic acid resulted in the enhancement of GSH, CAT, and SOD activities and a reduction in LPO at asignificant level. However, the effects were not purely dose-dependent except for the catalaseactivity (50x2 mg/kg, $p<0.05$; 100x2 mg/kg, $p<0.01$; 200x2mg/kg $p<0.001$).

The animals treated with different doses of rutin also significantly deviated the GSH, CAT, SOD activities and LPO levels, compared to the ZnO NP alone treated group. However,the observed effects were not completely dose-dependent. The lower and median dose of rutin imparted the effect at the significant levels $p<0.5$ and $p<0.01$, respectively. The higher dose-induced effects on GSH, CAT, SOD activities, and LPO levels were almost similar to those ofthe median dose with the same statistical significance ($p<0.01$). This indicates that rutin at its 100x2mg/kg b. wt. was the optimal dose to impart the maximum protective effect against the ZnO NP-induced adverse effects on biochemical parameters in the liver and testes. Treatment with the combination of L-ascorbic acid and rutin (each 100mgx2/kg wt) induced the ameliorating effect in terms of a significant enhancement in GSH, CAT, SOD, andreduction in the LPO ($p<0.01$) induced by ZnO NP.

There are only limited reports for studies on ZnO NP-induced antioxidant enzyme activity. The present observations of ZnO NP-induced reduction in the reduced glutathione (GSH), superoxide dismutase (SOD) Catalase (CAT) are in parallel with previous reports thatalso showed similar kinds of effects on *Oreochromis niloticus* and *Tilapia zillii* (Saddick *etal*., 2017). ZnO NP acts as an antioxidant and reduces oxidative stress as per some earlier reports on in vivo (Afifi *et al*., 2015; El-Bahr *et al*., 2020). It may be true for its lower dose. The observed significant deviations in the antioxidant enzyme activities and lipid peroxidationmay be attributed to the higher dose selected in the present study. Any nutraceutical including antioxidant molecules beyond higher doses act as prooxidant leading to causing oxidative stressthat in turn affects the antioxidant status of the tissues and enhance lipid peroxidation; theremany reports on this aspect (Chakraborthy *et al*., 2014; Giordano *et al*., 2020). It has been proposed that the

antioxidant impacts of certain food items in the regular diet offer an inconclusive explanation of the environment's mechanisms with the modulation of oxidative stress (Miranda-Díaz *et al*., 2020).

It has been well deduced for several years through various experimentations that interference in the spermatogenesis hampers the semen both in terms of quality (morphology and motility and quantity (sperm count) (Wyrobek and Bruce, 1978; Wan *et al*., 2013). In the present study, ZnO NP at its higher doses imparted a negative impact on epididymal sperm quality and density that were statistically significant. A similar kind of observation was also made in earlier studies (Abbasalipourkabir *et al*., 2015; Srivastav *et al*., 2017). The adverse effects may be due to inflammation also since an earlier study demonstrated that the ZnO NPs could induce inflammation in male germ cells (Braydich-Stolle *et al*., 2010). It has been reported that some nanoparticles including ZnO NP accumulated in the testes and subsequently crosses the blood-testis barriers (Chen *et al*., 2004; Borm and Kreyling, 2004).Thus, altogether including inflammation, free radical generation and DNA damage might have resulted in the observed effect on the sperm parameters. Based on the results of the enzyme and lipid peroxidation assays and DNA damage, and from earlier reports (Abbasalipourkabir *et al*., 2015), it can be speculated that the exposure to ZnO NP leads to the generation of some free radicals that can readily react with electronegative DNA molecule which in turn resulted in the formation of micronucleated cells, abnormal spermatozoa and reduction in sperm count and P/N ratio. The selected nutraceuticals ascorbic acid and rutin were found to attenuate these effects.

In conclusion, the ZnO NP was found to be capable of imparting toxic effects on somatic and germinal cells as indicated by bone marrow micronucleus assay and sperm parameters of Swiss albino mice. However, the observed effect was dose and time-dependent.The higher the dose, the greater was the magnitude of the adverse effects. The 24-hour time interval was found to be more effective and at later time intervals, there was a decline in the potency of the toxic effects as noted in the micronucleus test. The selected nutraceuticals, L- ascorbic acid, and rutin were found to be effective in reducing the toxicities caused by ZnO NP both individually and in combination. However, they did not completely neutralize the effectseven at the highest dose selected. The antioxidant enzyme assays and lipid peroxidation experiments confirmed the ZnO NP induced oxidative stress at its higher dose, which in turn might have led to the formation of micronuclei in bone marrow cells and DNA damage in spermatozoa. The Ames test results indicated that ZnO NP was not mutagenic; however, its higher concentration induced the base-pair substitution after metabolic activation. Therefore, though ZnO NP has many applications, caution must be taken to avoid over-exposure to it through various routes and means in view of circumventing health risks.

CHAPTER –III

Evaluation of Mutagenicity of Titanium Dioxide (TiO_2) Nanoparticle and Modulatory Effects of Nutraceuticals (L-Ascorbic Acid and Rutin)

3.1. Introduction and Review of Literature:

In recent years, nanotechnology is a subject that gained so much research interest to derive advantages in many fields. Titanium dioxide (TiO_2 NPs) is one of the very commonly utilized nanoparticles. TiO_2 is a natural oxide of the element titanium and is considered to havelow toxicity and insignificant biological effects (Textor *et al*., 2001; Grande and Tucci, 2016).The utilities of TiO_2 depend upon particle size with special reference to ultrafine particles < 1 00 nm, and fine particles 0.1 to ca. 3 μm (Grande and Tucci, 2016).

The bio-inert material of normal-sized (>100 nm) TiO_2-
NPs) made it to be comprehensively used in various food products. In addition, it is used as an ingredien

t in a widerange of pharmaceutical products and cosmetics, including sunscreens and toothpaste (Ziental *et al*., 2020; Baranowska-Wójcik, 2020). TiO_2-
NPs have been reported to possess an antibacterial property that is exploited to extend the shelf life of f oodstuffs (Rhim *et al*., 2013).

With reference to studies on the mutagenicity of TiO_2, there are few reports. TiO_2 NPshave been demonstrated to exhibit genotoxicity and impair DNA repair activity in A549 cells (Jugan *et al*., 2012). Shukla *et al*. (2011) reported that ROS-
mediated genotoxicity induced TiO_2 NPs in human epidermal cells. The persistence of TiO_2 NPs - induced genotoxicity has been estimated in mice (Mohamed *et al*., 2015). Cytotoxicity and genotoxicity of shape-engineeredTiO_2-NPs have been demonstrated in bronchial epithelial cells (Gea *et al*., 2019).

It has been reported that the main mechanism underlying the potentially toxic effects of TiO_2 is a matter of its chemical and physical characteristics such as size, crystal structure, and photo-activation. Therefore, in view of the widespread applications of TiO_2 NP, and potential exposure, the present study was carried out to evaluate the mutagenicity of nano-sized particles of TiO_2.

3.2. Materials and Methods:

Chemicals and Reagents

Titanium (iv) oxide, anatase, NP, <25nm particle size (CAS No: 1317-70-0), purchased from Sigma Aldrich, Bengaluru, India was used as the test chemical.

Details of all other chemicals and reagents used for the experiments were as given in chapter 2 (section 2.2.1).
The details of the bacterial strains, preparation of mouse liver microsomal enzymes (S9 fraction); Bacterial reverse mutation assay (Ames test); in vivo studies using Swiss albino mouse as the experimental model (Bone marrow Micronucleus Test; Epidydimal Sperm Parameters; Sperm Chromatin Dispersion (SCD) test); Biochemical Parameters (Catalase activity; Superoxide dismutase (SOD) assay; Reduced Glutathione (GSH) Assay; Lipid Peroxidation Assay), and statistical analysis are as given in the chapter (section 2.2).

The dose and treatment schedules:

TiO_2 nanoparticles were selected with the three doses (50, 100, and 200 mg/kg b.wt.) based for the in vivo studies based on earlier reports on its pharmacokineticsin animal models (Geraets *et al*., 2014; Warheit *et al*., 2015;). For the study, 3 healthy animalswere given an i.p. injection of TiO_2 NP with a dose of 50 mg/kg b.wt and observed for 7 days for any adverse effects. No toxic symptoms were observed and the doses were selected for the experimentations.

The toxicities of nanoparticles were assessed by constituting the experimental animal groupsas follow s:
Group I: Solvent control administered with HPMC
Group II: Low dose group - TiO_2 NP (50 mg/kg b.wt)
Group III: Median dose group - TiO_2 NP (100 mg /kg b.wt).
Group IV: High dose group - TiO_2 NP (200 mg/kg b. wt.)
Group V: Distilled water control for cyclophosphamide (CP)
Group VI: Positive control group - CP (50 mg/kg b. wt.)
Group VII: Solvent control for multiple treatments with HPMC for 7 consecutive days.
Group VIII: Subchronic (multiple) treatment - TiO_2 NP for 7 consecutive days.

The aforesaid groups were taken for three-time intervals 24, 48, and 72 hours (in case of subchronic effect, sampling was done 24 hours after the last treatment). The data of solvent controls and positive control derived from the ZnO NP were used since the same ones were used for titanium dioxide toxicities assessment.

Since the micronucleus assay did not show a high level of genotoxicity for ZnO NP, the protective effect of the selected nutraceuticals was carried out using only sperm parametersin S wiss albino mice. The experimental groups were made as follows:

Group I: Animals treated with distilled water (solvent control for nutraceuticals)
Group II: Animals treated with L-ascorbic acid (200x2 mg/kg) treatment group
Group III: Animals treated with Rutin - 200x2 mg/kg
Group IV: Animals treated with TiO_2-NP 200 mg/kg b. wt. (the values derived from thetoxicity tests).
Group V: Animals treated with L-ascorbic acid (50X2 mg/kg) + TiO_2 -NP (200 mg/kg)
Group VI: Animals treated with L-ascorbic acid (100X2 mg/kg) + TiO_2-NP (200 mg/kg)
Group VII: Animals treated with L-ascorbic acid (200X2 mg/kg) + TiO_2-NP (200 mg/kg)
Group VIII: Animals treated with Rutin (50X2 mg/kg) + TiO_2-NP (200 mg/kg)
Group IX: Animals treated Rutin (100X2 mg/kg) + - TiO_2NP (200 mg/kg)
Group X: Animals treated Rutin (200X2 mg/kg) + TiO_2-NP (200 mg/kg)
Group XI: Animals treated with L-ascorbic acid + Rutin (100+100) x 2 (Control)
Group XI: Animals treated with [L-ascorbic acid + Rutin (100+100)x2] + TiO_2-NP (200mg/kg) + [VC + Rutin (100+100)mg/kg x2].

3.3. Results and Discussion:

3.3.2. Ames Test

The positive observations observed in the present Ames test were contradictory to earlier reports (Woodruff *et al*., 2012; Du *et al*., 2019) where TiO_2 nanoparticle was shown to be negative in different strains of *S. typhimurium*. The discrepancy in the results obtained maybe due to the concentration and size of the particles. Further studies need to be conducted to evaluate the mutagenic potency taking into consideration of various factors. From the present study, it was clearly noted that the selected TiO_2 NP was capable of inducing base-pair substitution and frameshift mutations. And, in the case of the nanoparticle, after metabolic activation, the mutagenic potency was enhanced.

3.3.2. Bone Marrow Micronucleus Assay

The induction of micronucleated cells by TiO_2 NP at multiple-dose treatment is in agreement with previous in vivo studies (Trouiller *et al*., 2009; Sycheva *et al*., 2011) studies. In these studies also TiO_2 NP showed genotoxic effects including micronucleus formation. Thus, from the current study with reference to the bone marrow micronucleus test, it can be deduced that acute exposure to TiO_2 NP up to 200 mg/kg bt. may not be genotoxic but multipleexposures even at a lower dose may cause genotoxicity.

3.3.3.Epidiymal Sperm Parameters

TiO_2 NP increased the frequency of total abnormal spermatozoa and DNA-damaged sperm cells at a significant level in the multiple dosing ($p<0.05$) in comparison with the contro lgroup. The lower and median doses did not significantly affect the morphology and DNA integrity of

sperm. The positive control CP induced the formation of a higher frequency of abnormal sperm ($p<0.01$) and DNA damage ($p<0.01$) at a significant level.

The single dose of TiO_2 NP significantly affected sperm morphology at 100 mg/kg ($p<0.05$) and 200 mg/kg ($p<0.01$). The multiple dosing (50x7 mg/kg) induced the effect with a highly significant level ($p<0.001$). With reference to the DNA damage in spermatozoa, TiO_2 NP induced the toxic effect statistically with a higher magnitude ($p<0.01$). The positive control CP-induced effect on sperm morphology and DNA integrity was evident fromthe data that significantly deviate from that of the control group ($p<0.001$).

TiO_2 NP at 24 hours exposure time did not induce a significant reduction in sperm count. However, there was a dose-dependent effect on motility as indicated by the significant reduction in the frequency of sperm with progressive motility (50 and 100 mg/kg, $p<0.5$; 200 mg/kg b.wt. $p<0.01$). The multiple treatment schedule affected the motility at a highly significant level ($p<0.001$). The positive control significantly affected the sperm count ($p<0.05$) and motility ($p<0.01$) significantly compared to its control group at 24 hours. The data of the animals exposed to the test chemical for 35 days duration indicated that the single exposure of the TiO_2 NP affected the count (100 and 200 mg /kg, $p<0.01$) and motility significantly in lower doses also. TiO_2 NP (single dose of 200 mg/kg and multiple dosing 50 mg/kg x7 days) resulted in a reduction in the count and motility at a highly significantlevel ($p<0.001$) compared to the HPMC control group. The positive control CP significantly affected the sperm count as well as motility significantly compared to its control group at 35 days of sampling ($p<0.001$).

Overall, TiO_2 NP was found to impart adverse effects on sperm parameters. The test chemicals more drastically affected sperm motility rather than the count. The TiO_2 Nanoparticle-induced negative effect on sperm parameters, including morphology, count, and motility was also demonstrated in mice (Trouiller *et al.*, 2009; Miura *et al.*,2019). Thus, the current observation of our study corroborates with the earlier reports.

Many earlier reports have shown that Leydig cells can uptake the titanium dioxide particles leading to inhibiting the viability and proliferation of these cells (Komatsu *et al.*, 2008).It has been demonstrated in the mouse model that TiO_2 NPs disrupt the blood-testis barrier byaltering the expression of several testis-specific genes (Hong *et al.*, 2015). All these reports indicate that testis is a target organ for TiO_2 NPs. confirmed that the testis is a target of TiO_2 NP.

L-ascorbic acid induced a decrease in the frequency of total abnormal sperm and, spermatozoa with DNA damage only at its higher dose (200x2 mg/kg) at a significant level ($p<0.05$) when compared with the TiO_2 NP treated group. The median and higher doses of L- ascorbic acid enhanced the sperm count with the same statistical significance ($p<0.05$). The progressive motility was found to be dose-dependent (100x2mg/kg, $p<0.05$; 200x2mg/kg, $p<0.01$). Thus, L-ascorbic acid exhibited some attenuating effect against TiO_2-induced adverseeffects on sperm quality and quantity, for which the threshold dose required was found to be 100x2mg/kg b.wt. Rutin imparted the ameliorating effect on TiO_2 NP-induced changes on sperm morphology, DNA integrity, sperm count, and motility only at its higher dose ($p<0.05$). However, its median dose showed a recovery effect on sperm morphology at a significant level ($p<0.05$). Overall, rutin 200x2 mg/kg was found to be a threshold to impart maximum protection against the adverse effects on sperm parameters induced by TiO_2 NP. The TiO_2 NP (200) + [Ascorbic acid + Rutin (100+100)x2] treated group changes in values obtained for sperm abnormality ($p<0.01$), DNA damage ($p<0.05$), sperm count ($p>0.05$)and progressive motility ($p<0.05$) compared to the TiO_2 NP induced values. This indicated thatthe combination of the nutraceuticals was more effective in imparting protection against sperm morphology compared to other parameters. Further, the combination of L-L-ascorbic acid andrutin was not found to be more effective compared to the individual treatment in protecting against TiO_2 NP-induced toxicities in spermatogenesis.

Biochemical Parameters

The positive agent CP (50mg/kg b.wt) affected the liver and testes biochemical parameters by significantly deviating the values compared to the distilled control group ($p<0.001$). The selected nutraceuticals L-ascorbic acid and rutin at 200x2 mg/kg b. wt. (the maximum dose selected for the present study) individually and in combination (each 100x2 mg/kg b.wt) did not change the GSH, CAT, SOD, and LPO levels at a significant level compared to the distilled water control group. Thus, the selected doses of the nutraceuticals aresafe with reference to the biochemical parameters in the Swiss albino mice. The solvent control (HPMC) also did not significantly alter the biochemical parameters.

The TiO_2 NP (200mg/kg b. wt)-treated group showed a significant decline in the GSH, CAT, and SOD activities and an increase in the LPO in both the tissue, viz., liver and testes. Among the detoxifying enzymes, SOD was found to be more significantly reduced ($p<0.01$) compared to the CAT and GSH ($p<0.05$) in both tissues compared to the HPMC control. The highly significant increase in the LPO values compared to the control group ($p<0.001$) indicated that TiO_2 NP induced lipid peroxidation with a higher magnitude.

The coadministration of TiO_2 NP with L-ascorbic acid resulted in the enhancement of GSH, CAT, and SOD activities and a reduction in LPO at a significant level. However, the effects were not purely dose-dependent except for the LPO (50x2 mg/kg, $p<0.05$; 100x2 mg/kg,$p<0.01$; 200x2mg/kg $p<0.001$). L-ascorbic acid at its 100x2 mg/kg b.wt. can be considered as an optimal dose yielding the maximum ameliorating effect against TiO_2 NP-induced toxicities in the liver and testes as indicated by statistical significant values (both 100x2 and 200 mg/kg b.wt.) at the level of $p<0.01$ compared to the TiO_2 alone treated group.

The animals treated with different doses of rutin also significantly deviated the GSH, CAT, SOD activities and LPO levels, compared to the ZnO NP alone treated group. However, the observed effects were not completely dose-dependent. The lower dose of rutin (50x2mg/kg)was not found to be effective in reducing the toxicities except for GSH activity. The intensity of the protective effect of rutin against TiO2-induced changes in biochemical parameters was found to be oscillating between the tissues. Overall, it can be seen that rutin at the dose of 200x2 mg/kg b. wt. was found to be more effective compared to the lower doses in imparting the protective effect as indicated by statistically significant values in GSH, SOD, and LPO ($p<0.01$). Treatment with both the nutraceuticals L-ascorbic acid and rutin (each 100mgx2/kg b.wt.) induced the ameliorating effect in terms of a significant enhancement in GSH, CAT, SOD,and reduction i n the LPO ($p<0.01$) induced by TiO2 NP. TiO_2 NP-induced changes in biochemical parameters, including detoxifying enzyme activities, have been reported in an earlier study (Hong *et al*., 2015). There are many reports on TiO_2 NP imparting free radicals/oxidative stress-induced adverse effects (Bhattacharya *et al*., 2009; Hu *et al*., 2019). In the present study also, it was observed drastic changes in defensive enzyme activities, particularly Superoxide dismutase activity, and also lipid peroxidation in thetestes in response to TiO_2 NP treatment. Thus, a moderate decline in the TiO2 NP-induced testicular toxicities may be attributed to the antioxidant potentiality of the selected nutraceuticals. L-ascorbic acid was found to be more effective compared to rutin in bringing about the ameliorating effects on TiO_2 NP-induced adverse effects on sperm parameters.

In conclusion, TiO_2 particles having varied applications and widespread exposures were found to cause mutation, particularly base-pair substitution as indicated by *S. typhimurium* TA100 strain, and the effect was enhanced after metabolism activation. The *in vivo* studies on somatic (bone marrow) and germinal cells indicated that the testis is a target organ for TiO_2 where it may interfere with spermatogenesis leading to negatively affecting sperm quality and quantity. It was also found to induce DNA damage as noted from the sperm-chromatin dispersion test and structures of testes as indicated from the histological observation.The testicular toxicities in terms of sperm abnormality, reduction in sperm count, progressively motile sperm, and sperm with DNA damage could be attenuated substantially using nutraceuticals such as L-ascorbic acid and rutin owing to their potential antioxidant property. Thus, large and long terms exposures to TiO_2 nanoparticles have to be minimized to avoid health risks and

dietary supplements with antioxidants such as L-ascorbic acid and rutin may be helpful in minimizing its adverse effects.

CHAPTER – IV

Evaluation of Mutagenicity of Methyl Parathion andAntimutagenicity of the Nutraceuticals – A Study in *Salmonella typhimurium* TA100

4.1.Introduction and Review of Literature

Pesticides play an important role in the modern agricultural system. Methyl parathion (MP), an organophosphorus insecticide, reported having adverse effects on non-target organisms, including humans. As human beings are continuously exposed to these toxic chemicals through various means and routes, it is essential to study the mutagenic potency of these chemicals. Methyl parathion (MP) is an organophosphorus pesticide, widely used to control insect pests by acting as an inhibitor of acetylcholine esterase. It is a thiophosphoric acid ester with an empirical formula $C_8H_{16}NO_5POS$. It is mainly used to control boll weevils, and many biting or sucking insect pests of agricultural crops. Since it reaches non-targetorganisms, including human beings, addressing its various adverse effects is also important.

MP has been subjected to bacterial reverse mutation assays and found to be positive interms of inducing base-pair substitution (Rashid and Mumma,1984; Coral M N,2009). Mutagenicity and carcinogenicity are strictly interlinked. Taking into consideration its inevitable role in agriculture on one side and harmful effects on the other hand, some remedies have to be found to prevent or minimize the adverse effects of such toxic agents in non-target organisms).

Considering the potential antioxidant property of L-AA and rutin, it was selected in the present study to elucidate its possible protective role against the mutagenicity induced by an organophosphorus pesticide (Methyl parathion).

4.2.Materials and Methods:

Chemicals:

Metacid-50, a commercial grade of methyl parathion (O,O-dimethyl-O-4-nitrophenyl phosphorothionate; CAS No. 268-00-0), manufactured by Bayers India Ltd., Mumbai was usedfor the experiment.
Sodium azide (NaN3; CAS No. 26628-22-8) purchased from HiMedia Laboratories, Pvt. Ltd., Mumbai, India was used as the positive mutagen. All other chemicals were procured from Merck, SRL, and Himedia, India.

The protective effect of rutin was assessed at 5, 10, and 20 mg/plate against 0.5 mg/ml of MP-induced mutagenicity with and without metabolic activation.

Rate of antimutagenicity = [(M-S1)/ (M-S0)] ×100 where, M is the number of revertant colonies/plate induced by mutagen alone, S0 is the number of spontaneous revertant colonies, S1 is the number of revertant colonies/plate in the presence of the extract and the positive mutagen (Agar *et al*., 2010).

Bacterial Strains: *Salmonella entrica typhimurium* (strain: TA100; MTCC Acc. No. 1252) was procured from Microbial Type Culture Collection and Gene Bank, CSIR-Institute of Microbial Technology (IMTECH) Council of Scientific and Industrial Research (CSIR), Chandigarh, India. Further details as given in section 2.2.2.

Preparation of Mouse Liver Microsomal Enzymes (S9 fraction): Explanation as given in section 2.2.3.

Bacterial mutagenicity assay: Explanation as given in section 2.2.2.

Statistical analysis: As detailed in section 2.2.3.7.

4.3. Results and Discussion

In the present study, the mutagenesis assay was carried out with three concentrations of MP i.e., 0.1, 0.5 and 1.0 mg/ml. Among the concentrations taken, a concentration-dependent increase in the number of CFU was observed for 0.1 ($p<0.01$) and 0.5 mg/mL ($p<0.001$) of MP, indicating its mutagenic potency in terms of base-pair substitution. It was noted that MP without metabolic activation (-S9 fraction) and with metabolic activation (+S9 fraction) imparted the toxicity. However, in the presence of the S9 fraction, mutagenicity was of a higher magnitude compared tothat of the MP in the without S9 fraction ($p<0.05$).

A similar type of results was obtained in earlier studies (Rashid and Mumma, 1984; Coral, 2009). Thus, it can be inferred fromthe results that MP by itself can impart the mutation (base-pair substitution) and the effect was augmented after it was metabolized in the liver. MP, being a phosphorus-containing alkylating agent can readily impart the mutation as it can form the DNA adduct (DNA interstrand cross-links) (Zeiger, 2010). It has been proposed that DNA alkylation leads to nucleobase modifications, attributed to the potential mutagenic effects (Ojha and Gupta., 2017). The enhancement of mutagenicity after mouse liver metabolic activation as observed in the present study in terms of increase in the CFU may be due to the additive effect.

The results of the antimutagenicity study for rutin against MP without S9 are presented in bar diagram and it can be seen that there was no significant change (decrease) in the number of revertants compared to that of the MP (0.5mg/mL). It indicates that rutin could not attenuatethe MP-induced base-pair substitution in TA-100. Thus, it can be inferred that rutin did not have an affinity toward MP blocking its DNA-adduct formation/covalent binding. However,there was a concentration-dependent decline in the CFU compared to that of MP co-incubatedwith S9 fraction and rutin at significant levels compared with the MP alone incubated culture.($p>0.01$ for 5.0mg/mL and $p>0.001$ for 10.0 mg/mL). However, the highest concentration of rutin tested was found to impart antimutagenicity with the same magnitude ($p>0.001$) as that of the lower concentration.

A similar kind of observation was also made in an earlier study where a further increase in the concentration did not enhance the antimutagenic activity (Mader and Macdonald, 1985). This is an indication of threshold concentration; further, an increase in the concentration of rutin beyond 10

mg/mL against MP (0.5 mg/mL) did not enhance the antimutagenicity. It has been reported that MP is bioactivated by the P450-dependent microsomal mixed-function oxidase system and by microsomal GSH-transferases, and enhancesthe DNA binding capacity (Snijman *et al*., 2007).
As specified earlier, MP has been reported to generate free radicals and impart oxidative stress-induced mutation. Thus, based on the presentobservation and earlier reports, it can be inferred that rutin bearing a potent antioxidant propertycould attenuate the free radical-mediated mutation induced by MP.

The current observation andinference are in parallel with an earlier study (Bartoli, 1991) wherein, the antimutagenic activities of rutin in *Salmonella*/ reversion assay, against peroxide mutagens, were attributed toradical scavenging effects. It has been proposed that the carbonyl group at C4 and the presenceof the 2,3-double bond in the C-ring play a crucial role in the antimutagenic activity of flavonoids (Mader and Macdonald, 1985). Rutin is a flavonoid with a similar structure, which may be one of the plausible reasons for the observed modulatory effect against MP-induced base-pairsubstitution.

Rutin has been reported to be a nontoxic and non-oxidizable bio-flavanoid unlike other flavonoids (Batra and Sharma, 2013) which made us select this molecule for assessment of its possible modulatory effect against a potent genotoxic agent, MP. Three different concentrations of rutin, viz, 5mg/ml, 10mg/ml and 20 mg/ml were assessed for their mutagenicity and antimutagenicity. All the tested concentrations of rutin were not found to impart mutagenicity before and after metabolic activation as indicated by the number of CFU almost similar to that of the negative control. Further, unlike MP, it did not induce the lethal effect even at the highest concentration selected. The non-mutagenicity of rutin was also reported in an earlier study. Thus, the concentrations of rutin selected are safe as far as mutagenicity and lethal effect on *S. typhimirium* TA-100 strain.

Thus, it can be concluded that rutin which has been exploited for its role in a protectiveeffect against various toxicants is further confirmed in this study. It was found to impart the threshold-dependent modulatory effect against the mutation (base-pair substitution) induced by MP after metabolic activation.

L-Ascorbic acid alone did not show any increase in the number of revertants both in the presence and absence of the S9 fraction, compared to the solvent control.It indicates that it is not mutagenic up to 20.0 mg/mL in the *S. typhmurium* TA 100, which made us select the L-AA at concentrations of 5.0, 10.0, and 20.0 mg/mL. A comparison between MP (0.5mg/mL) and co-treatment with L-AA showed a significant reduction in the number ofrevertant colonies compared to the MP alone treated plate. The combined treatment significantly reduced the number of revertant colonies; L-AA at its lower concentration (5.0 mg/mL) reduced the number of colonies at a significant level ($p<0.05$). At a higher concentration of L-AA (10.0 mg/mL), the reduction rate was significantly enhanced up to 36.36% ($p<0.001$). Though the highest concentration of L-AA reduced the number of colonies compared to the MP alone treated plates, the rate of reduction was almost similar to that of thehigher dose ($p<0.001$). It indicates that L-AA at its 10.0mg/mL is the threshold concentration to yield the maximum antimutagenic activity against MP as noted in the present observation. A similar trend was also observed in the Ames test with the S9 fraction. The percentage increase in the inhibitory effect in the case of the S9 fraction indicated that L-AA imparted antimutagenic activity after metabolic activation of MP or L-AA or both.

The exact mechanism(s) underlying the inhibitory effect of VC against MP-induced mutagenicity is not fully understood. However, the antimutagenic potential of VC against different chemicals including organophosphorus pesticides has been demonstrated in many invitro and in vivo systems (Geetanjali *et al*., 1993; Kuzhir *et al*., 1999). A number of diverse mechanisms of genoprotective action of VC against a wide variety of chemicals have been suggested (Edgar, 1974; Bijur *et al*., 1997;

Odin, 1997; Durnev, 2018). It has been advocated that VC exerts its effects through multiple inhibition mechanisms (De Flora and Ramel, 1988).

MP-induced mutagenic effects may be due to the higher alkylating potential of methylesters. MP contains the phosphorothioate (P=S), which is oxidized to oxon (P=O), methyl- paraoxon, by liver microsome oxidase in the liver. The oxons are highly toxic compounds, which account for the profound cytotoxic effect of organophosphorus pesticides (Vijayaraghavan and Nagarajan, 1994). Under physiological conditions VC effectively competes with cellular nucleophilic sites, such as DNA, RNA or proteins, thus masking these sites from binding by the mutagen (Edgar, 1974; Kaya, 2003). This aspect of VC activity has been demonstrated in other studies where the vitamin prevented or decreased the binding of carcinogens to macromolecules including DNA (Bodo *et al*., 1989; Surh *et al*., 1994). Thus, the antimutagenicity of VC against MP can be related to its ability to block MP and/or its metabolites from covalent binding to cellular DNA. The pesticide undergoes a series of enzyme-mediated biotransformations in the liver. Therefore, one of the bases for mutation, it is these biotransformed products act as mutagens. Hence, the decrease noted in the mutagenicity of MP in the Ames test, treated concurrently with L-AA, may be due to the L-AA- mediated hydroxylation of lipid/cholesterol, thus concomitantly minimizing the changes in theformation of mutagenic conjugates of the pesticide with them.

L-AA, since it is as hydrophilic in nature, it is the most importantfree-radical scavenger in extracellular fluids, trapping the radicals in the aqueous phase and protecting the biomolecules, including DNA (Altuntas *et al*., 2002). Probably, the enhanced protective effect of L-AA as observed in the present study may be due to the scavenging of free radicals generated from MP during its metabolic activation.

Thus, whatever the mode(s) of action of L-AA in minimizing the mutagenicity of the pesticide (MP), its protective role appears to be evident enough. Based on this, it can therefore be suggested that vitamin C is a good supplement to avoid/minimize the potential health risksdue to exposure to toxic agents such as MP.

CHAPTER – V

Antimutagenic Activity of Ethanolic Extract of *Roccella montagnei* against Sodium Azide – An *In Vitro* Study

5.1. Introduction and Review of Literature:

According to a recent estimate, the incidence of cancer is expected to increase by more than 75% by the year 2030 in developed countries, and over 90% in developing nations (Bray*et al*., 2012). Increased environmental pollution and modern lifestyle-related chemical exposure are the main reasons for various health disorders, particularly cancers. Since a number of chemicals released to the environment are innumerable, practically it is not possible to completely avoid exposure to all mutagens/carcinogens. In addition, some of the agents, particularly therapeutics are life-saving, which cannot be withdrawn from their utility, despite they are known to induce mutation. Therefore, there is always a good demand for agents, which can minimize chemicals-induced genotoxicity and carcinogenicity. Natural sources are a repository of various molecules with potential medicinal properties.

Lichens are considered to be a good source of various bioactive molecules as they produce a variety of different secondary metabolites in response to environmental stress conditions (Rankovi *et*

al., 2015). The significance of lichens and their metabolites, including various medicinal properties has been reviewed (Huneck, 1999).

Roccella montagnei Bel. Emend. A, belonging to the family Roccellaceae is commonly found as epiphytes along the Coromandel coast, Tamil Nadu, India, and is also abundant in Pichavaram mangrove forests (Awasthi, 1988; Balaji *et al*., 2006). Polar and non-polar solventextracts of *R. montagnei* have been screened for various biological activities such as anti microbial (Balaji *et al*., 2006; Anjali *et al*., 2014; Saklani *et al*., 2016; Ferreria and Silva, 2021), mosquito larvicidal & antifungal (Nanayakkara *et al*., 2005), insecticidal against *Helicoverpa armigera* (Balaji *et al*., 2007) and anti-inflammatory (Vijayakumar *et al*., 2000) activities. However, there are no studies so far reported for the antimutagenic activity of this particular lichen. The present work was aimed at evaluating the ethanolic extract of *R. montagnei* (EERM) for its mutagenic and antimutagenic activity against sodium azide (NaN3), a potent mutagen and also a carcinogen, by employing the Ames test with *Salmonella typhimurium* TA100 strain, with and without metabolic activation (S9 fraction).

5.2. Materials and Methods:

5.2.1. Collection and identification of the lichen:

R. montagnei was collected from the Gonikoppa of Kodagu district, Karnataka, a part of the Western Ghats region in the month of October 2015. The lichen was identified based on morphological, anatomical and chemical te sts (Awasthi, 1988). Identification of the lichen wasauthenticated taking the help of scientists from Lichenology Laboratory, Plant Diversity, Systematics and Herbarium Division, CSIR-National Botanical Research Institute, Lucknow, India.

5.2.2. Preparation of Ethanolic Extract of the Lichen:

The collected lichens were shade dried at room temperature for 3 days. The dried lichens were chopped into pieces and powdered with the help of a mixer. A known quantity of the powdered lichen (50 g) was dissolved in 250 ml of distilled ethanol by using a Soxhlet apparatus for 8 hours at 78^{o}C. The extracts were filtered using Whatman No.1 filter paper and then concentrated in a vacuum at 40°C using a rotary evaporator, lyophilized and stored in a sterile container at 4°C until further use. The yield of the crude extract obtained was 6.2 % on a dry weight basis.

5.2.3. Bacterial strains: Details as given in section 2.2.4.

5.2.4. Preparation of the Mouse Liver Microsomal Enzymes (S9-fraction): Details as given in section 2.2.3.

Positive Mutagen:

Sodium azide (NaN_3; *CAS No.* 26628-22-8) purchased from HiMedia Laboratories,Pvt. Ltd., Mumbai, India was used as the positive mutagen. NaN_3 solution at a concentration of 1.0 µg/plate was freshly prepared during th e experiment by dissolving in 10% DMSO.

5.2.5. Mutagenicity and Antimutagenicity Testing :

As explained in the previous chapter the test was conducted based on the plate incorporation method described by Maron and Ames (1983) using the bacterial strain in the absence and presence of the S9 fraction.

5.2.6. Statistical Analysis: As detailed in section 2.2.3.7.

5.3. Results and Discussion:

Fig. 5.3.1. Mutagenicity and Antimutagenicity of EERM against NaN_3 with (+S9) and without (-S9) liver microsomal fraction

The results obtained for mutagenicity and antimutagenicity assays of EERM against NaN_3 with and without S9 fraction were presented in fig.5.3.1 The culture plates inoculated with NaN_3 showed more revertant colonies both in the absence and presence of S9 fraction, which were statistically significant compared to the solvent control ($p<0.001$). The current observation confirmed the mutagenic potency of the NaN_3 and is in accordance with the previous reports (Samiei *et al*., 2015; Akin *et al*., 2016). The number of revertants in the presence of the S9 fraction was found to be more when compared with the assay without the S9 fraction, indicating that products of NaN_3 after its metabolic activation induce base pair substation at a higher magnitude. Thus, it acts as both mutagen (carcinogen) and promutagen (procarcinogen) by inducing the point mutation, i.e., base pair substitution as indicated by *S. typhimurium* TA100, a special strain designed for indicating the base-pair substitution as observed in the present study and earlier reports (Issazadeh *et al*., 2012; Samiei *et al*., 2015; Akin *et al*., 2016).

Among the three concentration of the lichen extract selected, none of them, even the highest concentration did not induce the mutation as indicated by the number of revertants, which were almost equal to the negative control both in the absence and presence of the S9 fraction. It indicates that EERM neither by itself nor its metabolic products did not induce the base-pair substitution in *S. typhimurium* TA100. In other words, the ethanolic extract of the lichen does not contain any constituent responsible for the mutation in the DNA of the test organism. Thus,it can be said that the selected lichen is non-toxic as far as mutagenicity is concerned. The current observation, to some extent, is in parallel with the previous report (Agar *et al*., 2010), where two other species of lichen, *Rhizoplaca chrysoleuca* and *R. melanophthalma* were found to be non-mutagenic in *S. typhimurium* TA1537.

When the assays were performed for antimutagenicity against NaN_3-induced mutation, the number of revertant colonies was significantly reduced in the absence of the S9 fraction for the highest dose ($P<0.05$) as well as in the presence of the S9 fraction compared to the solvent control (5 µg/mL, $p<0.05$; and for 10 & 20 µg/mL, $p<0.01$). There was a dose-dependent inhibitory effect of the extract. However, the observed effect was more enhanced in the presence of the S9 fraction, where the rate of inhibition was found to be 18.74, 36.3 and 38.7 % with an increase in the concentration of the extract when compared with the assays without the S9 fraction, which were found to be 7.48, 11.3 and 22.0 %. The exact reason for the enhanced protective effect after metabolic activation is not clear. Probably, certain constituents of the lichen inhibit the metabolic products of NaN_3 from inducing the base pair substitution in the test organism. In antimutagenicity testing, according to Ong *et al*. (1986), the antimutagenic effect is considered strong when the inhibitory effect is above 40% and moderatewhen the effect is between 25-40%, and less than 25% is considered as weak. In accordance with this, the antimutagenicity of *R. montagnei* can be considered as weak (<22.0%) against NaN3 and moderate (>25%) against its metabolic products, as observed in the present study.

The exact mechanism(s) of EERM-imparted antimutagenicity against NaN_3 after its metabolism, is not known. Various constituents present in the *R. montagnei* (Rashmi and Garampalli, 2014) can decrease the rate of mutation by inactivating the mutagenic action or byinhibiting the metabolic activation of NaN_3. The chemical composition *R. montagnei* include erythrin, erythritol, lecanoric acid, roccellic acid, isolichenin, oricinol, β-

carotene and montagnetol (Rao and Sheshadri, 1940; Seshadri and Subramanian, 1949). Whether all these constituents imparted the antimutagenicity by synergistic action or a particular molecule is responsible for the observed antimutagenicity cannot be pointed out at this level. Among the constituents, β-carotene is well known for antimutagenic activity against certain agents based on the in vitro (Arriaga-Alba *et al*., 2000) and in vivo (Aidoo *et al*., 1995; Berti *et al*., 2014) studies.

Thus it can be concluded that *R. montagnei* is a good source of active principle(s), which can be exploited for the prevention or minimization of cancer risks caused by potential mutagenic agents like sodium azide.

Summary and Conclusions:

- The rapidly developing industrial sector has led to the use and release of a variety of toxic chemicals to the environment. Many pollutants that enter the biological system may induce mutation and associated health disorders.
- Toxicological studies help in preventing or reducing the likelihood that a disease or other negative health outcome would occur due to exposures to chemical agents.
- In recent years, nanoparticles have found their many applications and there are wide- exposures through various routes and means and attracted much research attention for their toxicity assessments.
- Direct or indirect exposures to chemicals became inevitable due to increased environmental pollution and modern lifestyles-related products that cannot be completely avoided. One of the remedial approaches is the usage/consumption of nutraceuticals to reduce health risks.
- In the present study, two nanoparticles namely titanium dioxide and zinc oxide nanoparticles were evaluated for their mutagenicity by employing the Ames test (bacterial reverse mutation assay) with or without mouse liver microsomal (S9) fraction.
- TiO_2 nanoparticles and a ZnO nanoparticle were evaluated for their dose (50, 100, and 200 mg/kg b. wt.) and time yield (24, 48, and 72 for micronucleus test and 24 and 35 days post-treatment sampling for sperm parameters) effects by administering i.p. injection to the experimental animals.
- A nutraceutical is a substance that has physiological benefits as well as provides protection against health disorders caused by various physical and chemical agents.
- L-ascorbic acid, a potent water-soluble vitamin and rutin a polyphenolic flavonoid present in many plants with potential antioxidant properties were selected as the nutraceuticals for their possible modulatory activity against the toxicities induced by the test chemicals.
- Swiss albino mouse was used as the experimental model and toxicity studies were conducted on somatic and germinal cells by employing bone marrow micronucleus testand sperm abnormality assay (morphology, count and motility), respectively, and alsohistopathological observation of testes.
- HPMC was used as the solvent control and Cyclophosphamide (50mg/kg b.wt.) was used as the solvent and positive control for toxicity studies for nanoparticles. Sodium azide and 10% DMSO were used as positive and solvent controls for the Ames test.
- In order to study the mechanism of actions for toxicity and protective effects of the nutraceuticals, antioxidant enzymes assays (reduced glutathione, superoxide dismutase, catalase activities); lipid peroxidation of liver and testes of the treated animals and DNA damage studies (sperm chromatin dispersion test) in the spermatozoa were carried out.
- The study was also conducted to evaluate the possible antimutagenic activity of ethanolic extract of a lichen *Roccella montagnei* against sodium azide, a potent mutagenic agent by employing bacterial reverse mutation assay in *S. typhimurium* TA100 strain (Ames test).
- The ZnO nanoparticle was found to be capable of imparting toxic effects on somatic and germinal cells as indicated by bone marrow micronucleus assay and sperm parameters of Swiss albino mice. A dose-dependent increase in the toxic effects was observed. The 24-hour time interval was found to be more effective and at later time intervals, there was a decline in the potency of the toxic effects as noted in the micronucleus test.

- The selected nutraceuticals, L-ascorbic acid, and rutin were found to be effective in reducing the toxicities caused by the ZnO nanoparticle both individually and in combination. However, they did not completely neutralize the effects even at the highest dose selected.
- The antioxidant enzyme assays and lipid peroxidation experiments confirmed the ZnONP induced oxidative stress at its higher dose, which in turn might have led to the formation of micronuclei in bone marrow cells and DNA damage in spermatozoa.
- The Ames test results indicated that ZnO NP was not mutagenic; however, its higher concentration induced the base-pair substitution after metabolic activation. Therefore,though ZnO NP has many applications, caution must be taken to avoid over-exposure to it through various routes and means in view of circumventing health risks.
- The TiO2 nanoparticle was found to cause mutation, particularly base-pair substitutionas indicated by *S. typhimurium* TA100 strain and the observed effect was more enhanced after metabolism activation.
- The in vivo studies on somatic (bone marrow) and germinal cells indicated that the testis is a target organ for TiO2 where it may interfere with spermatogenesis leading tonegatively affecting sperm quality and quantity. It was also found to induce DNA damage as noted from the sperm-chromatin dispersion test and histological observationof testis.
- The testicular toxicities in terms of sperm abnormality, reduction in sperm count, progressively motile sperm, and sperm with DNA damage could be attenuated substantially using nutraceuticals such as L-ascorbic acid and rutin.
- Exposures to TiO2 nanoparticles have to be minimized to avoid health risks and dietarysupplements with antioxidants such as L-ascorbic acid and rutin may be helpful in minimizing its adverse effects.
- Pesticides play an important role in the modern agricultural system. Methyl parathion(MP), an organophosphorus insecticide, is reported to impose various adverse effects onnon-target organisms, including mutagenicity.
- In the present study, the possible antimutagenic activity of L- ascorbic acid and rutin were assessed against MP-induced mutagenicity by employing the Ames test. *Salmonella typhimurium* –TA 100 was used as the test strain representing the base-pairsubstitution.
- A concentration-response toxicity was performed by selecting 0.1, 0.5, and 1.0 mg/ml of MP. The protective effect of rutin was assessed at 5, 10, and 20 mg/ml against 0.5 mg/ml of MP-induced mutagenicity with and without metabolic activation.
- The results indicated that MP induced concentration-dependent mutagenicity up to 0.5mg/mL However, growth inhibition was observed in the highest concentration of MP, both in the presence and absence of S9 fraction, indicating the lethality.
- Rutin induced a concentration-dependent decrease in the frequency of revertant colonies at a significant level up to 1.0mg/ml compared with that of MP-treated strainsin the presence of S9 fraction. Thus, rutin has the potency to minimize the base-pair substitution induced by MP after metabolic activation, with a threshold limit.
- L-ascorbic acid and rutin were found to possess the potency to minimize the base-pairsubstitution induced by MP after metabolic activation, with a threshold limit.
- The bacterial reverse mutation assay confirmed the high mutagenic potency of NaN_3 with or without S9 fraction. The ethanolic extract of *Roccella montagnei* imparted the antimutagenic effect at the moderate level against NaN_3 in thepresence of the S9 faction as indicated by a significant decrease in the number of revertants.

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Dr. Shyama Prasad Sajankila, Associate Professor, Department of Biotechnology, NMAM Institute of Technology, Nitte, Karkala, for providing laboratory facilities to carry out Ames test.

Dr. Sanjeeva Nayaka, Scientist, Lichenology Laboratory, Plant Diversity, Systematics, and Herbarium Division, CSIR-National Botanical Research Institute, Rana Pratap Marg, Lucknow - 226001, U.P., India, for helping in the identification of lichen and its preservaiton.

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Publications

Naik, P., Nair, A., Nayaka, S., &Sajankila, S. P. (2030). Antimutagenic Activity ofEthanolic Extract of *Roccellamontagnei*against Sodium Azide–An In Vitro Study.CryptogamBiodiversityandAssessmentVol1,No.2(2016),e-ISSN:2456-0251,89-92

Nair, A., &Naik, P. (2020). Rutin imparts attenuation of methyl parathion-inducedbase-pair substitution. *Research Journal of Pharmacy and Technology*, *13*(12), 5680-5684.

PresentationsintheConferences:

Presented a poster titled, "Mutagenicity of Zinc oxide nanoparticle using Ames testwithandwithoutmetabolicactivation",intheInternationalConferenceonEnvironment, Genes,Health and Diseases - 2017, organized by Bharathiar University,Coimbatore,on August22nd to24th,2017.

Presented a poster titled, "An in vivo assessment ofgenotoxicity of titanium dioxidenanoparticle", in the 6th International Science Congressorganized by Internationial Science Community Association atHutatmaRajaguruMahavidyalaya,Puneon 8-9thDec.2016.

Presented a poster titled "Genoprotective and cytoprotective effect of l-ascorbic acidagainst cyclophosphamide - a broad spectrum anticancer drug' in the UGC sponsoredNationalSeminaronCancer–MultidisciplaaryApproachorganizedbytheDepartmentofMolecularBiology,Kannuruniversity,atDr.P.K.RajanMemorialCampus,Nileshwar,KasargodonSeptember19-20,2014.

(Amritha Nair)
Research student

Place :Mangalagangothri
Date : 21.12.2021

(Dr. PrasanthaNaik)
Research supervisor

Place :Mangalagangothri
Date : 21.12.2021

Dr. Prashan[illegible]a Naik M.Sc.,Ph.D
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	Suggestions given by the Committee	Actions taken
1.	To use proper standard format for references and ensure the same throughout. Many references cited in the text are not listed in the references list	A standard reference format is used and uniformity is maintained throughout. All missing references were added.
2.	The title as well as the objectives to be made more scientific to the work carried out.	The objectives are reframed in accordance with the title of the thesis and are now more scientific.
3.	The rationale for the work needs to be brought out by justifying from gaps in the literature. Specifically, use of two nanoparticles, one pesticide and one lichen extract needs to be justified.	Justifications for the works carried out are now given with the supporting literature. As per the titles, chemicals agents of two categories (nanoparticles and pesticide) were selected based on environmental exposures to them. Justification for lichen extract for antimutagenicity study is given.
4.	The work carried out pertaining to the objectives is vast. However, the last objective proposed, that is molecular mechanism was not fulfilled.	Parameters such as sperm-DNA integrity test, enzymes and micronucleus assays, Ames test give insight into the molecular mechanisms of toxicity and protective role of nutraceuticals.
5.	To present objective-specific, focused literature review, including recent literature between the latest quoted 2015 and current year, that is 2021.	Recent references upto 2021 are now added to make it more focussed on objectives.
6.	The data has been subjected to statistics, so F/t statistic, df and Post hoc test where applicable have to be mentioned.	All the experimental data were analyzed with appropriate statistical tools.
7.	Rationale to be provided for the doses of nanoparticles, doses of nutraceuticals as well as use of only ethanolic extract for the lichen study.	Rationale for the dose selections for the test chemicals and nutraceuticals is given. Justification for ethanolic extract of the lichen is given.
8.	The methodology part in the synopsis did not include the nanoparticles, pesticides, lichen or even the nutraceuticals protocols used.	A brief explanation for all the experimental parameters are given in the methodology chapter.
9.	The synopsis submitted did not tally with the presentation.	I will present it so that content in the report and ppt presentation would tally.
10.	As presentation is in the PPT mode, methodology, particularly of the experimental groups may be presented graphically	It will be followed during the presentation
11.	Results to be presented either as graphs or as tables, not both. Graphs and tables should be self explanatory and of adequate size to ensure visibility in PPT.	It will be followed during the presentation

(Amrita Nair)

MANGALORE UNIVERSITY
Institutional Animal Ethics Committee

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Prof. K. Byrappa
Honourable Vice-Chancellor,
Mangalore University,
Mangalagangothri – 574 199.

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Date: 23.09.2015

Cert. No. MU/AZ/50/(C)/IAEC/2015-2016.

To:

Ms. Amritha Nair
Department of Biosciences,
Mangalore University.

Certificate

This is to certify that, your proposal entitled "*Assessment of Certain Environmental Agents for Mutagenicity and the Possible Role of Genoprotective Effects of Nutraceuticals*" submitted to IAEC was discussed in the meeting held on 23.09.2015 and committee has approved the proposal. You are permitted to use **135 numbers of Swiss albino mice** for the said project during the period of two years commencing from September 2015.

Dr. N.A. Madhyastha
Main Nominee, CPCSEA

Dr. N. A. Madhyastha
"INCHARA"
CHITPADI, UDUPI - 576 101
Karnataka, (N.D.)

Prof. K. Byrappa
Chairman

BHARATHIAR UNIVERSITY

DEPARTMENT OF HUMAN GENETICS AND MOLECULAR BIOLOGY
COIMBATORE - 641 046, INDIA

INTERNATIONAL CONFERENCE ON
ENVIRONMENT, GENES, HEALTH & DISEASES - 2017
22nd - 24th August 2017

CERTIFICATE

This is to certify that ~~Prof~~/ ~~Dr~~/ ~~Mr~~/ Mrs/~~Ms~~ AMRITHA NAIR
has ~~participate~~d/presented a paper - ~~Oral~~/Poster/~~acted as Session Chair~~
entitled " MUTAGENICITY OF ZINC OXIDE NANOPARTICLES USING AMES TEST WITH AND WITHOUT METABOLIC ACTIVATION "
in the scientific deliberations of "INTERNATIONAL CONFERENCE ON ENVIRONMENT, GENES, HEALTH & DISEASES - 2017" organized by the DEPARTMENT OF HUMAN GENETICS AND MOLECULAR BIOLOGY, Bharathiar University, India during 22nd - 24th August 2017.

CME 20 CREDITS

Dr. V. BALACHANDAR	Dr. A.VIJAYA ANAND	Dr. B.VANITHA	Dr. A.GANAPATHI
ORGANIZING SECRETARY INTL. CONFERENCE - EGHD 2017	HEAD OF THE DEPARTMENT HGMB, BU, INDIA	REGISTRAR i/c BU, INDIA	VICE - CHANCELLOR BU, INDIA

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|| MARUTI ||

6th International Science Congress

(ISC-2016)

8th & 9th December, 2016

Organized by

International Science Community Association

(Registered under Ministry of Corporate Affairs, Government of India)

in collaboration with

Hutatma Rajguru Mahavidyalaya, Rajgurunagar, Pune, Maharashtra, India

Certificate

This is to certify that Mrs. Amritha Nair has participated in 6th **International Science Congress** (ISC-2016) on 8th & 9th December 2016 held at Hutatma Rajguru Mahavidyalaya, Rajgurunagar, Pune, Maharashtra, India and presented a paper (oral/poster) entitled "An in Vivo assessment of Genotoxicity of Titanium Dioxide Nanoparticle"

He/She has been awarded

Prof. Dipak Sharma	Dr. S. B. Patil	Dr. Verla Andrew Wirnkor	Prof. Yogaranee Sakthivel
Coordinator ISC-2016 Professor and Head Maharaja Ranjit Singh College Indore, MP, India	President ISC-2016 Principal Hutatma Rajguru Mahavidyalaya, Rajgurunagar Pune, Maharashtra, India	Convenor ISC-2016 Senior lecturer, Department of Chemistry, Imo state University, Imo State, Nigeria	Secretary ISC-2016 Senior lecturer, Department of Educational Psychology, University of Colombo, Colombo, Sri Lanka

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DEPARTMENT OF MOLECULAR BIOLOGY

DR. P.K. RAJAN MEMORIAL CAMPUS, NILESHWARAM, KASARAGOD-671 314.

UGC SPONSORED NATIONAL SEMINAR

ON CANCER-A MULTI DISCIPLINARY APPROACH

19-20 SEPTEMBER 2014

CERTIFICATE

This is to certify that/Prof./Dr./Sri./Smt. AMRITHA NAIR, Research Scholar
Dept of Post Graduate Studies & Research in Biosciences, Mangalore University *has Participated /Presented a paper in the UGC SPONSORED NATIONAL SEMINAR ON CANCER-A MULTI DISCIPLINARY APPROACH on 19-20 September 2014 at the Department of Molecular Biology, Kannur University, Dr. P.K.Rajan Memorial Campus, Nileshwaram, Kasaragod, Kerala.*

Dr. A.M. Sreedharan
Campus Director &
Course Director (In charge)

Sri. Sanoop.P
Seminar Co-ordinator